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Ultrasound-assisted biosynthesis of novel methotrexate-conjugates

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ABSTRACT

New methotrexate-acylglycerols and methotrexate-cyclodextrins (α, β and γ-CD) conjugates were obtained via esterification or transesterification reactions. All reactions were catalysed by esterases namely immobilized Lipase from *Candida antarctica B* and Lipase from *Thermomyces lanuginosus*. The use of ultrasound to assist the reactions revealed to be a key factor to obtain high conversion yields on both MTX conjugates. Transesterification reactions including long chain triacylglycerols were only successful when ultrasound was applied. In cyclodextrins esterification a higher number of MTX molecules was also linked to cyclodextrins when ultrasound was used. All the conjugates were characterized by MALDI-TOF and NMR spectroscopy.

1. Introduction

The production of drugs as esters derivatives is still the most common strategy of the pharmaceutical industry to improve their physicochemical, pharmacokinetic or biopharmaceutical properties [1]. The synthesis of prodrugs can overcome problems related with chemical instability, rapid pre-systemic metabolism, toxicity, etc [1]. Moreover, the active principal of the drugs is not affected since it can be restored by esterases present in the blood, liver and other tissues [2].

Methotrexate (MTX) is an anticancer drug, used in the treatment of choriocarcinoma, lymphocytic leukemia, lymphomas and some solid tumours [3]. It also presents therapeutic uses for rheumatoid arthritis and some autoimmune diseases [4]. Despite its relevant properties this drug shows toxicity mainly related to dose side effects and drug resistance of the tumor cells [3]. The synthesis of methotrexate prodrugs has revealed to overcome some resistance problems associated to cancer cells [5]. Wu et al., have synthetized methotrexate-proline prodrug by solid-phase peptide synthesis methodology. They were able to overcome the resistance of MDA-MB-231 cells, being the prodrug more active than methotrexate [5]. Kuznetsova et al., produced methotrexate conjugated with a diglyceride unit as a prodrug. The conjugate was incorporated in the bilayer of a liposomal formulation. These liposomes bearing the methotrexate prodrug were able to overcome the resistance of human leukemia cells [6].

Lipases are well recognised efficient enzymes with high chemo, regio- and enantiospecific specificity and selectivity [7]. As catalysts in esterification/transesterification reactions, lipases can be applied in the presence of organic solvents [8], ionic liquids [9], aqueous medium [10–13], or in solvent free conditions [14], proving to be versatile assets. Nevertheless, any lipase-catalysed process is dependent on factors such as stability, mass transfer, selectivity, among others [15]. The enzymatic catalysis assisted by ultrasound has been proved to overcome some of the negative aspects associated with the reactional processes using lipases. The presence of ultrasound improves the enzymatic reactions comparing with the reactions performed its absence, by improving the mass transfer, enhancing the substrate dissolution, minimizing the reactional time, increasing the reaction yield and the chemo-, regio- and stereo selectivities [16–18]. An example, is reported by Gumel et al., that used Lipase from *Candida antarctica B* for the polymerization of ε-caprolactone to poly-6-hydroxyhexanoate by ring opening method. The ultrasound-assisted reaction showed to convert 75 % of the monomer while a conversion of only 16 % was observed with the conventional process [19].

Triacylglycerols are composed by a glycerol moiety, linked by ester bonds to three carbon chains. They are present in plants and in food, being essential to the human organism [20]. The formation of a prodrug containing an ester unit of a triacylglycerol can be considered as a novel methodology to produce non-toxic prodrugs.

Cyclodextrins are cyclic-oligosaccharides commonly used for the solubilization of hydrophobic compounds in aqueous medium by the

Abbreviations: MTX, Methotrexate; TL, Lipase from *Thermomyces lanuginosus*; CALB, Lipase from *Candida antarctica B*; US, Ultrasound; CD, Cyclodextrins; WB, Water bath

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formation of inclusion complexes. The complexation of a cyclodextrin with a drug is a methodology already used by the pharmaceutical industry [21]. Cyclodextrins are nontoxic macromolecules, and after complexation with a drug, they improve its solubility and stability, increasing its bioavailability, and reducing its toxicity [21].

Shin and co-workers [22] reported other application for these cyclic-oligosaccharides. They used a lipase as catalysts for the esterification reaction of oleic acid with *n*-butanol by ultrasonication in buffer medium. They proved that the addition of cyclodextrins as emulsifier compounds allowed higher solubilization of the hydrophobic compounds affording excellent yields (ŋ = 80 %) of the ester product.

The promising results obtained by Gumel et al. [19] and Shin et al. [22], regarding the green biosynthesis assisted by ultrasound lead us to explore similar conditions for the production of new MTX-acylglycerols and MTX-cyclodextrins conjugates via transesterification and esterification reactions. The enzymatic catalysis was performed using two lipases, namely immobilized *Candida antarctica B* and Lipase from *Thermomyces lanuginosus*. An aqueous/biphasic medium was used, enabling higher solubilization of the reagents and higher catalytic activity of the lipases. The transesterification reactions were performed using the following triacylglycerols: Glycerol Tributyrate (C_3), Glycerol Trivalerate (C_4), Glycerol Trihexanoate (C_5), Glycerol Tristearate (C_{17}) and Triolein (C_{17} with unsaturated bond at C_8). The esterification reactions were performed using cyclodextrins (α, β and γ-cyclodextrin). All the new conjugates were characterized by MALDI-TOF and ^{1}H and ^{13}C NMR spectroscopy.

2. Materials and methods

2.1. Materials and equipment

Commercial *Candida antarctica Lipase B* (CALB), Fermase CALBTM 10,000, immobilized on glycidyl methacrylateter-divinylbenzene-terethylene glycol dimethacrylate was obtained as a gift sample from Fermenta Biotech Ltd., Mumbai, India (activity of 8000 propyl laurate U/g). Lipase from *Thermomyces lanuginosus*, solution, ≥100,000 U/g was purchased from Sigma-Aldrich. All other compounds used in this work were purchased from TCI Chemicals or Sigma-Aldrich and used without any further purification. Ultrafiltration was performed with Ultracel 1 kDa ultrafiltration discs, composed of regenerated cellulose, 47 mm (Millipore) with ultrapure water (Milli-Q). Melting points were determined using a Gallenkamp apparatus.

2.2. General procedure

2.2.1. MTX-acylglycerol (small carbon chain) conjugates

In a flask with MTX (30 mg) was added 200 μL of water, followed by the triacylglycerol (1 eq.). Immobilized CALB (3 mg, 10 % w/w) was added and the flask placed in an ultrasonic bath (USC600TH, VWR International Ltd., USA; frequency 45 kHz and power of 120 W) with duty cycles of 5 ON/5 OFF during a total time of 30 min. The enzyme was removed by filtration, and the water removed by lyophilization. Crude products were obtained as yellow solids and their characterization can be found in the supplementary data.

In the water bath approach, the same procedure was followed, but instead of ultrasound, a water bath (Grant, OLS Aqua Pro) operating at 40 °C, 100 rpm, was used during the same time period as in the US.

2.2.2. MTX-acylglycerol (big carbon chain) conjugates

In a flask with MTX (30 mg) was added the triacylglycerol (1 eq.) followed by 500 μL of Lipase from *Thermomyces lanuginosus*. The flask was placed in an ultrasonic bath with duty cycles of 5 ON/5 OFF during a total time of 30 min. The suspension was extracted with $CHCl_3$ (3 ×) for the removal of unreacted triacylglycerol, and the aqueous phase centrifugated using vivaspin 10 kDa (Sartorius) for the removal of the lipase. After freeze-dried an orange oil/solid product was obtained and their characterization can be found in the supplementary data.

In the water bath approach, the same procedure was followed, but instead of ultrasound, a water bath operating at 40 °C, 100 rpm, was used during the same time period as in the US.

2.2.3. MTX-cyclodextrin conjugates

In a flask with MTX (3 eq.) was added the cyclodextrin (30 mg) followed by 500 μL of Lipase from *Thermomyces lanuginosus*. The flask was placed in an ultrasonic bath with duty cycles of 15 ON/5 OFF during a total time of 2 h. The enzyme was removed by centrifugation using a vivaspin 10 kDa. The separation of unreacted MTX was performed by ultrafiltration (membrane disc of 1 kDa). The solution was freeze-dried to obtain the yellow solid products and their characterization can be found in the supplementary data.

In the water bath approach, the same procedure was followed, but instead of ultrasound, a water bath operating at 40 °C, 100 rpm, was used during the same time period as in the US.

2.3. Nuclear magnetic resonance spectroscopy

^{1}H and ^{13}C NMR spectroscopy were performed using a Bruker Avance III 400 (400 MHz for ^{1}H and 100 MHz for ^{13}C). DMSO-d_6 (Cortecnet) was used as deuterated solvent, and the peak solvent was used as internal reference.

2.4. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)

MALDI-TOF mass spectra were acquired on a Bruker Autoflex Speed instrument (Bruker Daltonics GmbH) equipped with a 337 nm nitrogen laser. The matrix solution for MALDI-MS measurement was prepared by dissolving a saturated solution of 2,5-dihydroxybenzoinc acid (DHB) or α-cyano-4-hydroxycinnamic acid (CHCA) in an aqueous solution of 0.1 % trifluoracetic acid (70 %) and acetonitrile (30 %). Samples were spotted onto a ground steel target plate (Bruker part n° 209519) and analysed in the linear positive or linear negative modes using factory-configured instrument parameters suitable for a 0–5 kDa *m/z* range (ion source 1: 19.5 kV; ion source 2: 18.3 kV). Time delay between laser pulse and ion extraction was set to 130 ns, and the laser frequency was 25 Hz.

2.5. Electrospray ionization (ESI)

Electro spray ionization was performed in a mass detector Thermo Finnigan LxQ (Linear Ion Trap). The analysis was made using a mass detector susceptible of analysis in full scan mode, SIM and MS/MS with positive ionization. The mass spectra range was between 50 and 2000 *m/z* and a capillary voltage of 29 V was used.

3. Results and discussion

3.1. Methotrexate-acylglycerol conjugates

The catalytic activity of both, immobilized CALB and liquid Lipase from *Thermomyces lanuginosus*, against different triacylglycerols (carbon chain length between C3 and C17) was investigated using a water and

Table 1
Experimental conditions and conversion yields for methotrexate-acylglycerol conjugates using immobilized CALB and Lipase from *Thermomyces lanuginosus* (*TL*) (results with standard deviation from at least 2 independent experiments).

Entry	Triacylglycerol	Carbon Chain Length	Reaction Conditions	Yield (%)**	
				Immobilized CALB	Lipase *TL*
1	Glycerol Tributyrate	C3	WB (40 °C, 30 min)	–*	–*
2			US (5 min ON/5 min OFF; 30 min)	61.4 ± 4.0	–*
3	Glycerol Trivalerate	C4	WB (40 °C, 30 min)	–*	–*
4			US (5 min ON/5 min OFF; 30 min)	64.1 ± 0.4	–*
5	Glycerol Hexanoate	C5	WB (40 °C, 30 min)	45.8 ± 1.7	–*
6			US (5 min ON/5 min OFF; 30 min)	58.0 ± 7.6	–*
7	Triolein	C18:1	WB (40 °C, 30 min)	–[a]	–*
8			US (5 min ON/5 min OFF; 30 min)	–[a]	62.6 ± 4.3
9	Glycerol Tristearate	C18	WB (40 °C, 30 min)	–[a]	–[a]
10			US (5 min ON/5 min OFF; 30 min)	–[a]	63.7 ± 6.4

[a] No reaction occurred.
* Only vestigial amount of conjugate product was detected by ^{1}H NMR.
** The yield was calculated based on the initial number of moles and the moles of the product.

an ultrasonic bath. The immobilized form was chosen for comparison due to its higher thermal stability and catalytic activity comparing with the free form. Moreover, the purification steps can be simplified using this enzyme form since a simple paper filtration removes the enzyme from the reactional medium [16].

The transesterification reactions were conducted herein using a green methodology, with water as solely solvent. MTX is poorly soluble in almost all the solvents, namely DMSO which does not allow the fully solubilization of the reactants disabling the hydrolytic activity of the esterases. The addition of the triacylglycerol to the MTX solution formed, as expected, an immiscible biphasic system, being the powder enzyme suspended at the soluble phase.

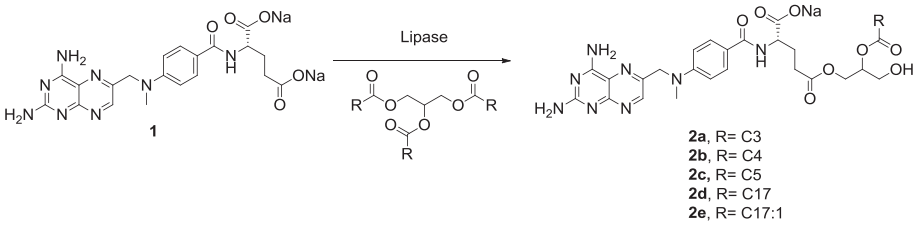
After reaction we observed that for longer triacylglycerols, namely triolein ($C_{17:1}$) and glycerol tristearate (C_{17}), immobilized CALB did not presented any hydrolytic activity (Table 1). After removal of the reactional mixture from the US, followed by a liquid-liquid extraction of the unreacted triacylglycerols, only free MTX was recovered in the aqueous phase. This result was expectable since recently, Chiplunkar et al. reported that immobilized CALB did not hydrolyse triolein in organic medium [23]. For the short triacylglycerols used, glycerol tributyrate (C_3), glycerol trivalerate (C_4) and glycerol trihexanoate (C_5), after 30 min under ultrasonication, we observed the disappearance of the biphasic system formed initially, and the precipitation of a solid that turns into soluble when more water is added to the reactional flask. The different catalytic behaviour of immobilized CALB, depending on the size of the triacylglycerols, may be justified by the poor mobility of the immobilized enzyme form to catalyse longer substrates. Considering this enzyme drawback, we replaced the immobilized CALB by a liquid Lipase from *Thermomyces lanuginosus* and use it without the addition of any other solvent. After reaction, it was possible to observe the hydrolysis of the longer triacylglycerols and the formation of the MTX-acylglycerol conjugates. Our data corroborate previous findings which highlight an high activity of this lipase for long triacylglycerols, such as triolein [24].

The reactional scheme proposed by us for the conjugation of MTX with triacylglycerols is presented in Scheme 1. We propose that the enzyme starts by hydrolysing the triacylglycerol, forming an intermediate in the active center, with the final release of a fatty acid chain. This reaction occurs twice, which in turns the glycerol unit with only one side chain available. Then, the MTX binds to the active site of the enzyme, by the carboxylic group, forming an ester intermediate. The glycerol can undergo a nucleophilic attack, which led to the formation of the final conjugates.

^{1}H NMR data did not allow us to ensure which carbonyl group is involved on the reaction, we therefore propose the γ-position carbonyl group, since it is the most reactive carboxyl group of the glutamic acid portion [25].

From ^{1}H NMR data we can infer that both lipases hydrolysed two of the three fatty acid chains of the triacylglycerols. Works reported have been recognising that some lipases can possess 1,3-selectivities against triacylglycerols [26]. Taking into account this enzyme property and considering that only one carbon chain remained attached to the glycerol moiety, we propose that the carbon chain remains in the second position (Scheme 1).

The use of ultrasound to assist the enzymatic reactions allowed to obtain higher conversion rates comparing with the results obtained using a water bath, concerning its emulsifying ability. As can be depicted in Table 1, high yields of crude products were obtained as solids or oils after freeze-drying. The effect of ultrasound on enzymatic reactions enhancement has been attributed to several reasons. One of them are the mechanical effects of ultrasound which promote the mass transfer from the bulk solution to the enzyme. The collision of substrate molecules and the enzyme promote the reaction rate increment and the miscibility of the two reactional initial phases. Another reason relies on



Scheme 1. Reactional scheme of methotrexate-acylglycerols conjugates synthesis. Compound **2a–2c** were produced using US in the presence of immobilized CALB. Compounds **2d** and **2e** were produced using US and Lipase from *Thermomyces lanuginosus*. **2a** was produced using glycerol tributyrate, **2b** using glycerol trivalerate, **2c** using glycerol trihexanoate, **2d** using glycerol tristearate and **2e** using triolein.

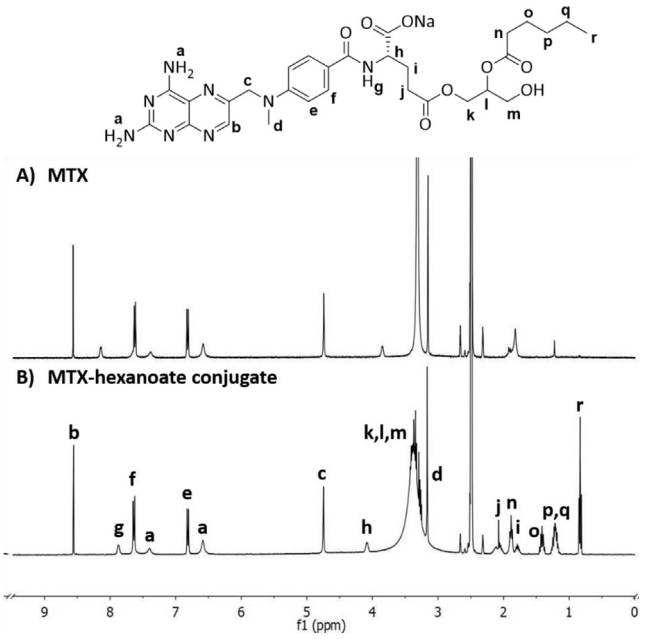


Fig. 1. ^{1}H NMR spectra of A) free MTX and B) methotrexate-hexanoate conjugate (DMSO-d_6).

the steady cavitation corrosion of ultrasound which might cause the enzyme molecule or cell granulation around it to be shared by micro-streaming [12], improving the mass transfer inside or outside of the enzyme. The final reason is related with the enzyme structure which in the presence of ultrasound become more flexible, and thus, may shift into its active configuration [18]. Considering that different enzymes have different stereo-configurations for the same ultrasonic parameters adopted, different enzyme behavior was observed for both immobilized CALB and liquid lipase. This distinct behavior is determined by several factors including the bulk of ultrasonic energy, the adaptability of the enzyme, and the micro-conditions around the enzyme. Analysing the entries **1–6** of Table 1, it is possible to observe that Lipase from *Thermomyces lanuginosus* was able to hydrolyse the short triacylglycerols in a low extent. The reaction yields calculated by ^{1}H NMR data were low revealing that this enzyme was only able to hydrolyse part of the carbon chains available. Only a vestigial amount of product was detected by the unfolding of the triacylglycerols peaks on ^{1}H NMR. High conversion yields were obtained on the conjugation of methotrexate with longer acylglycerols using the liquid enzyme form in the presence of ultrasound. In both cases, only vestigial amount of products was detected after incubation in the water bath. Herein, the use of ultrasound to assist the synthesis reactions was again crucial for an effective conjugation.

To confirm the conjugation, we also evaluated the melting point of the products produced. The melting point of methotrexate disodium salt is reported to be between 212 and 216 °C. For the conjugates produced we observed a different behaviour, they do not display a melting point, starting to decompose at around 250 °C. The conjugate composed by a hexanoate carbon chain is the most stable, starting to decompose at temperatures above 280 °C. The conjugate composed by a valerate chain (C4) is fully decomposed at 250 °C. The melting behaviour was not evaluated for the conjugates with longer carbon chains since they are isolated as a mixture of oil/solids.

^{1}H NMR spectra of conjugates **2a–e** (see Scheme 1) show a unique set of peaks of MTX protons, meaning that the transesterification reaction occurred in only one of the carbonyl groups (Fig. 1). The hydrolysed chains were eliminated during the liquid-liquid extraction (longer triacylglycerols) or during the freeze-drying process, in the case of short triacylglycerols. Fig. 1 shows the ^{1}H NMR of the methotrexate-hexanoate conjugate and of the free MTX. Significant chemical shifts are observed on the protons of methotrexate, mainly on the protons of the glutamic portion of the MTX. The amine protons signals remained untouched confirming that the conjugation occurred at one of the carboxylic groups.

The amide proton (*g*) suffers a chemical shift from δ_H 8.14 to 7.85 ppm. Proton *h*, appears at δ_H 4.08 instead of 3.85 ppm. The aliphatic protons of the glutamic portion unfold in two: proton *j* appears at a higher chemical shift δ_H 2.12 and 2.06 instead of 1.89 ppm; protons *i* stays in a similar place: from δ_H 1.79 to 1.82 ppm. Glycerol moiety is observed under the HDO peak, between δ_H 3.25–3.50 ppm.

Electro-spray ionization (ESI) and MALDI-TOF techniques were also assessed to confirm the formation of the conjugates (Table 2). The masses obtained either by ESI or MALDI-TOF are very similar to the theoretical values calculated, confirming the formation of conjugates between MTX and the acylglycerols.

Table 2
Values of theoretical mass and mass obtained by ESI and MALDI-TOF.

	Theoretical mass	Mass obtained by ESI	Mass obtained by MALDI-TOF
MTX-butyrate	620.6	666.6 (620.6 + 2Na$^+$)	625.4
MTX-valerate	634.2	625.5	631.4
MTX-hexanoate	648.6	659.2	671.1 (648.6 + Na$^+$)
MTX-olein	814.9	823.2	815.7
MTX-stearate	816.9	833.6	816.5

3.2. Methotrexate-cyclodextrin conjugates

Cyclodextrins (CD) are cyclic sugars used as devices for the solubilization of hydrophobic compounds by formation of inclusion-complexes in aqueous medium. Considering their non-toxic properties, the covalent binding of molecules to these devices can also be assessed as an effective strategy to produce pro-drugs [27].

We studied the biosynthesis of methotrexate-cyclodextrin

Table 3
Reaction conditions and conversion yields obtained after conjugation of methotrexate with cyclodextrins using liquid Lipase from *Thermomyces lanuginosus* (*TL*) (results with standard deviation from at least 2 independent experiments).

Entry	Cyclodextrin	Reaction Conditions	Lipase *TL*	
			Max. degree of modification	Conversion Yield
1	α-CD	WB (40 °C, 2 h)	1	8.9 ± 3.4 %
2	α-CD	US (15 min ON/ 5 min OFF) × 6 cycles	1	16.8 ± 1.8 %
3	β-CD	WB (40 °C, 2 h)	1	14.8 ± 3.4 %
4	β-CD	US (15 min ON/ 5 min OFF) × 6 cycles	2	19.0 ± 0.4 %
5	γ-CD	WB (40 °C, 2 h)	1	3.0 ± 4.2 %
6	γ-CD	US (15 min ON/ 5 min OFF) × 6 cycles	3	22.0 ± 2.8 %

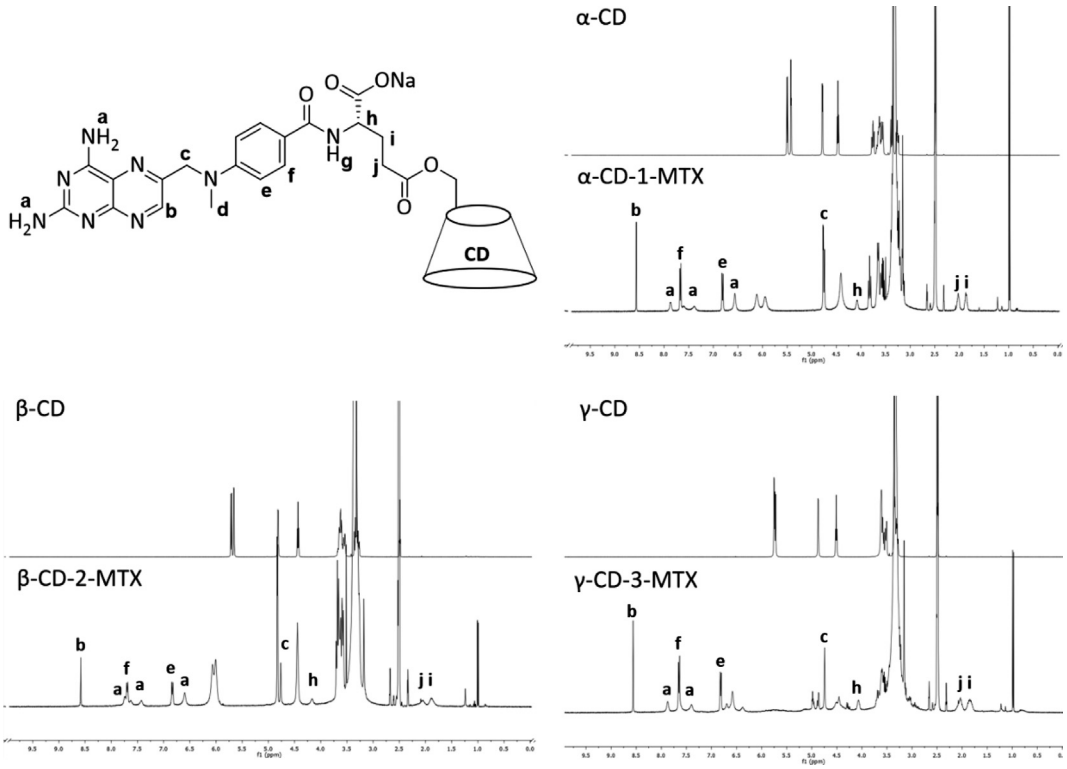


Fig. 2. ^{1}H NMR of MTX-CD (α, β and γ)-conjugates in DMSO-d_6 using Lipase from *Thermomyces lanuginosus* in the US.

conjugates using a similar methodology described previously for the triacylglycerols. The reactions were carried out using the liquid lipase from *Thermomyces lanuginosus* as reactional medium to ensure the solubilization of all the reactants, without addition of solvents. Immobilized CALB was also tested, however due to its difficulty to access and accommodate longer substrates an yield extremely low (around ŋ = 1 %) was obtained after reaction.

The effect of ultrasound on the formation of the bio-conjugates was also evaluated. The reactions were performed using an US bath, during 2 h with duty cycles of 15 min ON followed by 5 min OFF. The same reaction time was used for the reactions performed in a water bath (WB). Comparing with MTX-acylglycerol conjugates, the total time of the reactions was extended considering the size of the cyclodextrin substrates. As previously reported for MTX-acylglycerol conjugates, the use of US led to the achievement of high yields of the MTX-CD conjugates, and a higher number of MTX units linked to the macromolecules.

The amount of MTX equivalents used was also incremented but the findings revealed that it does not led to higher degrees of modification nor to higher conversion yields.

In Table 3 are depicted the results obtained after MTX-CD conjugation. Observing the data, we found that the degree of modification of the cyclodextrins is size dependent and greatly influenced by stereochemical impediments. The larger the size of the cyclodextrin molecule, higher is the number of MTX molecules bound. The smaller cyclodextrin used α-CD, composed by 6 glucose units, is only conjugated to one molecule of MTX, while the longer γ-CD, with 8 glucose units, was conjugated to a maximum of 3 units of MTX.

By ^{1}H NMR spectroscopy, it is possible to observe significant differences between the spectra of the starting reactants and of the final conjugates. The decrease and change in the chemical shift of the OH protons of the cyclodextrins are the most evident alterations. As starting materials, the OH peaks of the CD appear between δ_H 5.75 and 4.43 ppm. When conjugated, these peaks are observed between δ_H 6.58 and 4.45 ppm. Differences in the glycosyl peaks of the CD, mainly in the chemical shifts and pattern, were also detected. The pattern of the MTX in all the cyclodextrins remains very similar in all the conjugates obtained (Fig. 2).

MALDI-TOF analysis allowed us to calculate the degree of modification of the cyclodextrins with methotrexate (Fig. 3). We confirm a direct correlation between the size of the cyclodextrin and the degree of modification. α-CD presented the lowest modification, with only one MTX unit (m/z = 1478) (**3A**), β-CD is conjugated with 2 units of MTX (m/z = 2128) (**3B**), and γ-CD is conjugated to 3 MTX units (m/z = 2692) (**3C**). Glycosyl cleavage can be observed in the MALDI-TOF spectra, with values around $m/z \approx 170$. Based on the ^{1}H NMR and MALDI-TOF data we propose in Fig. 3 the structure of the final MTX-CD conjugates.

4. Conclusions

In the present study we developed new methotrexate-acylglycerols and methotrexate-cyclodextrins (α, β and γ-CD) conjugates via enzymatic transesterification or esterification reactions assisted by ultrasound. We verified that ultrasound played a crucial role on the final conversion yields and degree of modification. The ultrasonic system was not only advantageous over the traditional water bath (WB) in terms of reaction rates, but was also responsible for the products purity and selectivity, namely for the isolation of cyclodextrins with high amount of MTX units conjugated. The therapeutic association of non-toxic triacylglycerols and cyclodextrins, are herein presented as promising therapeutic compounds since they may prevent the development

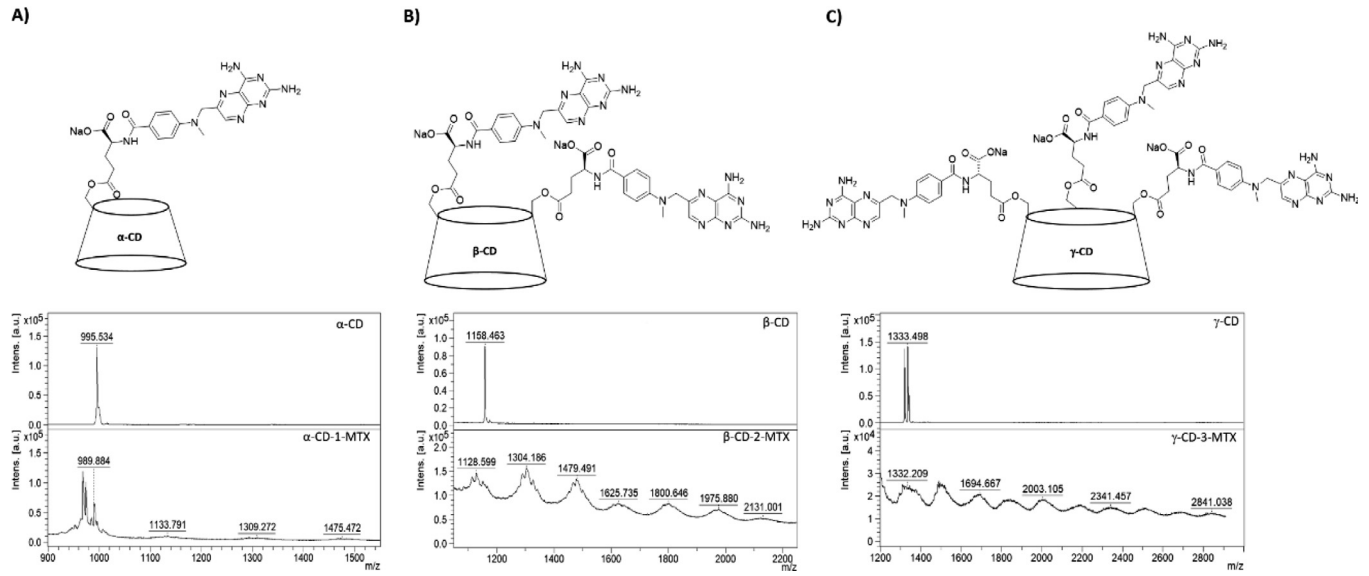


Fig. 3. MALDI-TOF of MTX-CD conjugates: A) α-CD conjugated with 1 MTX unit, B) β-CD conjugated with 2 MTX units, C) γ-CD conjugated with 3 MTX units; the upper image shows the proposed MTX-CD conjugates; all conjugates were obtained after reaction using Lipase from *Thermomyces lanuginosus* in ultrasound.

of transport resistance of the drug, which is often observed during the clinical use of methotrexate.

Acknowledgments

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Author information

The authors declare no competing financial interest.

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